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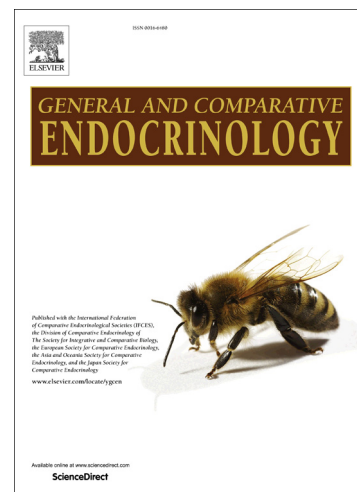
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Temperature induced variation in gene expression of thyroid hormone receptors and deiodinases of European eel (*Anguilla anguilla*) larvae

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Abstract

Thyroid hormones (THs) are key regulators of growth, development, and metabolism in vertebrates and influence early life development of fish. TH is produced in the thyroid gland (or thyroid follicles) mainly as T4 (thyroxine), which is metabolized to T3 (3,5,3'-triiodothyronine) and T2 (3,5-diiodothyronine) by deiodinase (DIO) enzymes in peripheral tissues. The action of these hormones is mostly exerted by binding to a specific nuclear thyroid hormone receptor (THR). In this study, we i) cloned and characterized *thr* sequences, ii) investigated the expression pattern of the different subtypes of *thrs* and *dios*, and iii) studied how temperature affects the expression of those genes in artificially produced early life history stages of European eel (*Anguilla anguilla*), reared in different thermal regimes (16, 18, 20 and 22°C) from hatch until first-feeding. We identified 2 subtypes of *thr* (*thra* and *thrβ*) with 2 isoforms each (*thraA*, *thraB*, *thrβA*, *thrβB*) and 3 subtypes of deiodinases (*dio1*, *dio2*, *dio3*). All *thr* genes identified showed high similarity to the closely related Japanese eel (*Anguilla japonica*). We found that all genes investigated in this study were affected by larval age (in real time or at specific developmental stages), temperature, and/or their interaction. More specifically, the warmer the temperature the earlier the expression response of a specific target gene. In real time, the expression profiles appeared very similar and only shifted with temperature. In developmental time, gene expression of all genes differed across selected developmental stages, such as at hatch, during teeth formation or at first-feeding. Thus, we demonstrate that the expression of *thrs* and *dios* show sensitivity to temperature and are involved in and during early life development of European eel.

Key words

Deiodinases, thyroid hormone receptors, fish, larvae, early life history

1. Introduction

The European eel (*Anguilla anguilla*) has been subjected to extensive scientific inquiry due to its enigmatic natural ecology (Van Ginneken and Maes, 2005; Miller et al., 2015; Righton et al., 2016), the critically endangered status of the population (Jacoby and Gollock, 2014), and the ongoing natural and anthropogenic pressures on the already historically low stock (Friedland et al., 2007; Bonhommeau et al., 2008; Geeraerts and Belpaire, 2010; ICES, 2015). As such, researchers are now employing technological means to circumvent the European eel decline and are striving to establish a sustainable aquaculture. Unfortunately, the aquaculture industry for European eel has been drastically reduced (ICES, 2015) due to the fact that it is capture-based, relying on wild-caught offspring (juvenile glass eels), which are unsustainably farmed until marketable size. The earliest life stages of European eel have not been encountered in nature, thus research needs to focus on laboratory studies to overcome current bottlenecks and gain knowledge about the species physiology and ecology. With recent advances in assisted reproduction (Butts et al., 2014; Müller et al., 2016; da Silva et al., 2016) and larval culture of European eel (Politis et al., 2014a; Sørensen et al., 2016; Butts et al., 2016) this is now possible and warrants further investigation. In addition, the European eel genome has recently been sequenced (Henkel et al., 2012), which offers new and emerging perspectives for fundamental molecular studies in eel biology (Rozenfeld et al., 2016).

In order to close the life cycle in captivity, similar bottlenecks needed to be overcome regarding the closely related Japanese eel (*Anguilla japonica*), which was accomplished in recent years (Masuda et al., 2012; Tanaka, 2015). Extensive efforts have been devoted to reach breakthrough achievements, forming the baseline knowledge for eel research (Tanaka et al., 2001, 2003). Subsequently, several studies have focused on assessing the extrinsic (environmental) and intrinsic (genetic) requirements and preferences of all life stages in order to identify the most

optimal conditions for rearing early life history (ELH) stages of this species (Yamano et al., 2007; Ahn et al., 2012; Hsu et al., 2015). However, still there seems to be large variation and differences in larval growth of artificially and naturally reproducing eels (Miller, 2009). Environmental factors such as temperature have been shown to drastically influence larval morphology and survival (Okamura et al., 2007; Ahn et al., 2012). Moreover, it was shown that the thyroid hormone (TH) pathway may control the early life development of Japanese eels, similar to Japanese conger eels (*Conger myriaster*) through thyroid hormone receptors (THR) (Kawakami et al., 2003; Kawakami et al., 2013). Thus, TH treatment has been applied to enable synchronized metamorphosis leading to a lower developmental variability (Yamano et al., 2007).

Generally, THs are key regulators of growth, development, and metabolism in vertebrates (Power et al., 2001, Warner and Mittag, 2012, Tata, 2006). THs are produced in the thyroid gland (or thyroid follicles) mainly as T₄ (thyroxine), which is metabolized to T₃ (3,5,3'-triiodothyronine) and T₂ (3,5-diiodothyronine) by deiodinase enzymes (DIO1-3) in peripheral tissues (Köhrle, 2000). The action of THs are mostly exerted by binding to specific nuclear THRs, although a non-genomic pathway has been shown to mediate TH functions through cellular signal transduction systems and cell surface receptors (Davis et al., 2008). THRs belong to the subfamily I of nuclear hormone receptors and share a modular structure composed of a N-terminal region (termed A/B domain), a conserved DNA binding domain (DBD or C region), a less conserved hinge region (D) followed by the ligand binding domain (LBD or E domain), and the C-terminal region (F region). In mammals, two genes (*thra* and *thrβ*) generally encode THR α and THR β (Lazar et al., 1993), whereas several genes encoding THR α and THR β can be found in fish, due to teleost genome duplication events (Nelson and Habibi, 2009). For instance, two *thra* genes have been described in zebrafish, *Danio rerio* (Takayama et al., 2008) and other fish species (Bertrand et al., 2007), while two *thrβ* have been reported in Japanese conger eel (Kawakami et al., 2003).

Increasing evidence suggests that THs have important roles during early life development and metamorphosis in fish (Marchand et al., 2004, Walpita et al., 2007, Infante et al., 2008, Campinho et al., 2010). The Japanese conger eel (Kawakami et al., 2003) and the Japanese eel (Kawakami et al., 2013) were previously chosen as model species to investigate the TH role on development and metamorphosis of Anguilliformes. An investigation of two isoforms of *thra* (*thraA* and *thraB*) and two isoforms of *thrβ* (*thrβA* and *thrβB*) showed differentially regulated gene expression for all isoforms during development and metamorphosis in Japanese eel (Kawakami et al., 2013). Thyroid hormones induce differential transactivation activity of the different *thr* isoforms, and interestingly, elevated gene expression was observed between hatching and the transition to exogenous feeding (Kawakami et al., 2013). Thus, it is of importance to further elucidate the *thr* expression profiles during this sensitive period of anguillid ELH, but especially during the still mysterious ELH of European eel.

The objectives of this study were to i) clone and characterize *thr* sequences, ii) follow the expression pattern of *thrs* and *dios*, and iii) investigate how temperature affects the expression of those genes in artificially produced ELH stages of European eel, reared in different thermal regimes from hatch until first-feeding.

2. Materials and Methods

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2012-15-2934-00458). Adult eels were anesthetized using ethyl paminobenzoate (benzocaine) before tagging and handling. Endogenously feeding larvae of European eel were

anesthetized prior to handling and euthanized prior to sampling by using tricaine methanesulfonate (MS-222). All efforts were made to minimize animal handling and stress.

2.1. Broodstock management and gamete production

Female silver eels were wild-caught (lake Vandet, Jutland, Denmark), while male eels were farmed (Stensgård Eel Farm A/S). Prior to experimental maturation, the fish were transported to a DTU Aqua research facility (Lyksvad Fishfarm, Vamdrup, Denmark), and housed in 300 L tanks equipped with a recirculation system (Tomkiewicz, 2012). Eels were maintained under low intensity light (~20 lux), 12 h day/12 h night photoperiod, salinity of ~36 ppt, and temperature of 20°C. Acclimatization took place over 14 days and the eels were not fed during the entire experimental period. Furthermore, eels were anaesthetized (ethyl p-aminobenzoate, 20 mg L⁻¹; Sigma-Aldrich, Missouri, USA) and tagged with a passive integrated transponder. To induce vitellogenesis females received weekly injections of salmon pituitary extract (Argent Chemical Laboratories, Washington, USA) at 18.75 mg kg⁻¹ body weight (Kagawa et al., 2005; Tomkiewicz, 2012). To stimulate follicular maturation and induce ovulation, females received 17 α ,20 β -dihydroxy-4-pregnen-3-one (Sigma-Aldrich, Missouri, USA) at 2 mg kg⁻¹ body weight (Ohta et al., 1996) and were strip-spawned after 12-14 h. Males received weekly injections of human chorionic gonadotropin (Sigma-Aldrich, Missouri, USA) at 150 IU/fish (Tomkiewicz, 2012). Prior to fertilization, an additional injection was given and milt was collected by strip-spawning ~12 h after administration of hormone. Milt samples were pipetted into a P1 immobilizing medium (Peñaranda et al., 2010) and only males with sperm motility of category IV (75-90%) were used for fertilization, within 4 h of collection (Butts et al., 2014). Eggs from each female were strip-spawned and fertilized separately by different sperm pools to create individual parental crosses.

Subsequently, 0.2 μm filtered UV sterilized North Sea seawater was added for a gamete contact time of 5 min. Seawater was adjusted to 20°C ($\pm 0.1^\circ\text{C}$) and 36 (± 0.1) ppt using Red Sea Salt (Red Sea Europe, Verneuil-sur-Avre, France). Only floating viable eggs/embryos were further used for experimentation.

2.2. Experimental conditions and design

During the spawning season, 25% of females successfully produced larvae after assisted reproduction treatments. Females represented here ($n = 4$) had a mean (\pm SEM) standard length and body weight of 65 ± 4 cm and 486 ± 90 g, respectively, while males ($n = 11$) had a mean (\pm SEM) standard length and body weight of 40 ± 3 cm and 135 ± 25 g, respectively. In this study, four individual parental crosses were used. Egg density for each parental combination was determined by counting 3×0.1 mL subsamples from the floating layer. Within 30 min post fertilization, ~500 floating viable eggs/embryos per 100 mL, with a mean size (\pm SD) of 1.5 ± 0.1 mm (at 2 hours post fertilization), were distributed in replicated 600 mL flasks [182.5 cm^2 sterile tissue culture flasks with plug seal caps (VWR[®])]. Seawater was supplemented with rifampicin and ampicillin (each 50 mg L^{-1} , Sigma-Aldrich, Missouri, USA), which previously has been shown to increase survival in a controlled experimental environment (Sørensen et al., 2014). Embryos and larvae, from each parental cross, were reared in thermally controlled incubators (MIR-154 Incubator, Panasonic Europe B.V.) at five temperatures (16, 18, 20, 22, and $24^\circ\text{C} \pm 0.1^\circ\text{C}$). All experimental units were acclimatized to the appropriate temperature regime within 1 h and salinity was kept at 36 ± 1 ppt. Temperature and salinity conditions were chosen to closely resemble the environmental conditions encountered at different depths of the assumed spawning areas in the Sargasso Sea (Castonguay and McCleave, 1987). Rearing of embryos and larvae took place in darkness, while handling and

sampling was conducted under low intensity ($< 2.2 \mu\text{mol m}^{-2} \text{s}^{-1}$) light conditions (Politis et al., 2014a). At hatch and every second day post-hatch until the corresponding first-feeding stage (Fig. 1), ~30 larvae from each parental cross ($\times 4$), each temperature (16, 18, 20 and 22°C), and each replicate ($\times 2$) were randomly sampled for later molecular analyses. Those endogenously feeding larvae were immediately euthanized using MS-222 (Sigma-Aldrich, Missouri, USA), rinsed with deionized water, preserved in a RNA Stabilization Reagent, and kept at -20°C following the procedure suggested by the supplier (Qiagen, Hilden, Germany). For further details on experimental design, rearing conditions, as well as larval morphology and development see Politics et al. (2017).

2.3. Data collection

2.3.1. Cloning and molecular characterization

cDNA sequences for *thrs* were predicted from genomic DNA sequences originating from the European eel genome assembly (WGS Project: AZBK01), available in NCBI resources (Henkel et al., 2012). Briefly, blastn requests were performed from Japanese eel cDNA *thr* sequences on the European eel WGS database to identify genomic contigs, including *thr* genes. European eel *thr* cDNA sequences were then deduced from contigs using GENSCAN (<http://genes.mit.edu/GENSCAN.html>) software (Burge et al., 1997). The predicted transcript sequences for each *thr* gene were confirmed by sequencing cDNAs from European eel RNA amplification. Cloning was achieved using TOPO TA cloning kit (Invitrogen) with pCRTMII-TOPO[®] vector, according to the manufacturer's instructions. Primers sequences were as follows:

thraA (F) 5'-GGGTCGCTGAGGAGACCT-3',

thraA (R) 5'-GGTGCTCAGACGTCCTGGT-3',

thraB (F) 5'-GCCCAACGACCCTAACCTAT-3',

thraB (R) 5'-GGATAGTAAATCAGGCCCTTAATC-3',

thrβA (F) 5'-GACTCCGAGGCCCTAATTCT-3',

thrβA (R) 5'-GACTCCGAGGCCCTAATTCT-3',

thrβB (F) 5'-GAAGACTGAGCCCTGAGGTG-3',

thrβB (R) 5'-TCACATGTACCCGCATTTGT-3'.

The cloned cDNA was sequenced with T7 and Sp6 primers by Beckman Coulter Genomics Inc (United Kingdom). European eel *thr* cDNA sequences have been deposited to GenBank® with accession numbers found in Table 1.

2.3.2. Phylogenetic analysis

A phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) with amino acid *thr* vertebrate sequences. The evolutionary distances were computed using the JTT matrix-based method (Jones et al., 1992) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. For the analysis, 1000 bootstrap replicates were carried out. Phylogenetic analysis was conducted in MEGA6 (Tamura et al., 2013). The GenBank accession numbers of the sequences used can be found in the Supplementary material.

2.3.3. Gene expression

For total RNA extraction, the larval pool (~30 larvae) of each replicate was homogenized in 800 µl Tri-Reagent (Sigma-Aldrich, Missouri, USA). After obtaining the aqueous phase by

incubation in 160 μ l chloroform, RNA was extracted using the InviTrap[®] Spin tissue RNA MiniKit (STRATEC Biomedical AG, Berlin-Buch, Germany) following the manufacturer's instructions. RNA concentration and purity were determined by spectrophotometry ($260/280 = 2.12 \pm 0.16$, $230/260 = 2.16 \pm 0.16$), using Nanodrop ND-1000 (PepLab, Germany). From the resulting total RNA, 680 ng were transcribed using the Quanta qScript cDNA Synthesis Kit (Promega, Germany), according to the manufacturer's instructions, including an additional gDNA wipe out step prior to reverse transcription [Quanta PerfeCta DNase I Kit (Promega, Germany)].

The expression levels of target genes (thyroid hormone receptors: *thraA*, *thraB*, *thr β A*, *thr β B* and deiodinases: *dio1*, *dio2*, *dio3*) and reference genes (*ef1 α* , *18s*, *40s*) were determined by quantitative real-time PCR (qRT-PCR) using specific primers. The *ef1 α* , *18s*, *40s* genes were chosen as housekeeping genes since qBase+ software revealed that these mRNA levels were stable throughout analyzed samples ($M < 0.4$); M gives the gene stability and $M < 0.5$ is typical for stably expressed reference genes (Hellemans et al., 2007). Using primer 3 software v 0.4.0 (<http://frodo.wi.mit.edu/primer3/>) primers were designed i) for *thraA*, *thraB*, *thr β A* and *thr β B* based on cloned cDNA sequences, ii) for *ef1 α* and *40s* based on sequences available in Genbank nucleotide database, and iii) for *dio1*, *dio3* and *18s* based on sequences available in the European eel transcriptome EelBase 2.0 database (<http://compgen.bio.unipd.it/eeelbase/>), as indicated in Table 1. To design the primers of *dio2* a European eel predicted cDNA sequence was used and the size of the amplicon obtained by amplification was checked (<http://www.zfgenomics.com/sub/eel>). All primers were designed for an amplification size ranging from 75 to 200 nucleotides and the amplicons obtained were of the expected size (Table1).

Expression of genes in each larval sample (4 parental crosses \times 2 replicates \times 4 temperatures) were analysed in technical triplicates using the qPCR BiomarkTM HD system (Fluidigm) based on 96.96 (GE chips) dynamic arrays (Miest et al., 2015). In brief, a pre-amplification step was

performed with a 500 nM primer pool of all primers in TaqMan-PreAmp Master Mix (Applied Biosystems) and 1.3 µl cDNA per sample at 10 min at 95°C; 14 cycles: 15 s at 95°C and 4 min at 60°C. Obtained PCR products were diluted 1:10 with low EDTA-TE buffer. The pre-amplified product was loaded onto the chip with SSofast-EvaGreen Supermix low Rox (Bio Rad) and DNA-Binding Dye Sample Loading Reagent (Fluidigm). Primers were loaded onto the chip at a concentration of 50 µM. The chip was run according to the Fluidigm 96.96 PCR protocol with a T_m of 60°C. The relative quantity of target gene transcripts was normalized against the geometric mean of the three housekeeping genes and analysed using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). Coefficient of variation (CV) of triplicates was calculated and checked to be < 0.04 (Hellemans et al., 2007). If CV was found to be > 0.04 , triplicates were checked for outliers and if possible duplicate measurements were used. If the use of duplicates was not possible ($CV > 0.04$) the whole data point was omitted from the analysis. Moreover, malfunctions on the chip and/or unsuccessful RNA extractions can result in expression failures, as was the case for some samples in this study.

2.4. Statistical analyses

All data were analyzed using SAS statistical software (version 9.1; SAS Institute Inc., Cary, North Carolina). Residuals were tested for normality using the Shapiro–Wilk test and homogeneity of variances was tested using a plot of residuals versus fit values (PROC GLT, SAS Institute 2003). Data were \log_{10} or arcsine square-root-transformed when data deviated from normality and/or homoscedasticity (Zar, 1996).

Statistical models were used to investigate temperature effects on gene expression throughout early larval development [Ages 0 to 18 days post hatch (dph)] and at specific developmental stages

(Stages 1-3). Across the different temperature treatments, Stage 1 represents the day of hatch, Stage 2 represents the timing of teeth formation, and Stage 3 represents the first-feeding stage (Fig. 1). Together, this allowed us to decipher changes in temperature at real-time and standardized developmental intervals.

To examine the effect of temperature on gene expression throughout early development (real time), we used two statistical approaches. In the first approach, we analyzed the data using a series of mixed-model repeated measures ANOVAs (PROC MIXED; SAS Institute 2003). Models contained the Temperature (16, 18, 20 and 22°C) and Age (0 to 18 dph) main effects as well as the Temperature \times Age interaction. Akaike's (AIC) and Bayesian (BIC) information criteria were used to assess which covariance structure (compound symmetry, autoregressive order, or unstructured) was most appropriate (Littell et al., 1996). Temperature and Age were considered fixed, whereas parental cross was considered random. Tukey's post-hoc analyses were used to compare least-squares means between treatments. If a significant Temperature \times Age interaction was detected the model was decomposed into a series of reduced ANOVA models to determine the effect of Temperature for each Age and of Age for each Temperature. This was the case for *dio3* and *thraA*. The reduced models involved only preplanned comparisons and did not include repeated use of the same data, so a-level corrections for a posteriori comparisons were not necessary.

In the second approach, we examined variation in gene expression, throughout development at each temperature, by fitting either linear, quadratic or cubic equations to the data (PROC REG; SAS Institute 2003). This allowed us to create predictive models to explore the shape of variation throughout development for each temperature. Linear, quadratic and cubic equations were chosen a-priori to fit the data based on the available literature (e.g. McDonald, 2009; Sørensen et al., 2016). Final equation selection (linear, quadratic or cubic) was based on an F-statistic: $d.f._j \times (R^2_j - R^2_i) / (1 - R^2_j)$, where: R^2_i = the R^2 for the i-th order, R^2_j = the R^2 for the next higher order, d.f._j = the degrees

of freedom for the higher-order equation with j degrees of freedom in the numerator and $d.f.j = n - j - 1$ degrees of freedom in the denominator (McDonald, 2009). Graphs and regressions were prepared in SigmaPlot (Version 13.0).

3. Results

3.1. Cloning and molecular characterization

In this study, two forms of THR α (termed aaTHR α A and aaTHR α B) as well as two forms of THR β (named aaTHR β A and aaTHR β B) were cloned in European eel larvae. The full CDS (coding DNA sequence) of the four THR α s was sequenced. The nucleotide sequences of aaTHR α A (GenBank accession number KY082904) and aaTHR α B (accession number KY082905) are 1375bp and 1334 bp long, with an open reading frame (ORF) that codes for a protein of 416 and 435 amino acids respectively, while the nucleotide sequences of aaTHR β A (GenBank accession number KY082906) and aaTHR β B (GenBank accession number KY082907) are 1288bp and 1299bp long, coding for 396 and 378 amino acid proteins respectively (see Supplementary material).

3.2. Phylogenetic analysis

The phylogenetic tree constructed by the Neighbor-Joining method from multiple sequence alignment of *thr* amino acid sequences from a range of vertebrates reveals that European eel *thra* and *thr β* , grouped into two clearly separated clades (99 bootstrap for both clades). In addition, the topology of the phylogenetic tree showed that European eel *thr* sequences clustered within the

teleost group and each time the Japanese eel homolog *thr* sequence was the evolutionary closest (Fig. 2).

3.3. Gene expression

No larvae survived at 24°C. Gene expression of selected genes was compared across the other temperature treatments (16, 18, 20 and 22°C) in real time and at specific developmental stages (developmental time). The expression of target genes was affected in real and/or developmental time by larval age (or stage), temperature, and/or their interaction (see specific genes below).

3.3.1 Deiodinases

In developmental time, expression of *dio1* significantly ($P = 0.004$) increased on Stage 2 (teeth formation) and 3 (first-feeding), while no significant ($P = 0.271$) effect of Temperature was detected (Fig. 3A-B). In real time, gene expression of *dio1* was significantly affected by Temperature ($P = 0.034$) and Age ($P < 0.0001$), while no significant Temperature \times Age interaction was detected ($P = 0.903$). Gene expression of *dio1* significantly increased at 22°C (Fig. 3C) and significantly increased throughout ontogeny with increasing larval Age (Fig. 3D). The relationship between Age and *dio1* expression was best explained by a sigmoidal cubic regression at 16°C (Fig. 3E). There were no significant relationships between Age and *dio1* expression (Fig. 3F-H) at the other temperatures.

Similarly, expression of *dio2* significantly ($P = 0.008$) increased on Stage 3 (first-feeding), while no significant effect of Temperature was detected ($P = 0.178$; Fig. 3I-J) in developmental time. In real time, gene expression of *dio2* increased throughout ontogeny with increasing larval

Age ($P < 0.0001$; Fig. 3L), while no significant Temperature ($P = 0.088$; Fig. 3K) or Temperature \times Age interaction ($P = 0.394$) was detected. The relationships between Age and *dio2* expression were best explained by sigmoidal cubic regressions at 16°C, 18°C and 20°C (Fig. 3M-O). There was no significant relationship between Age and *dio2* expression at 22°C (Fig. 3P).

On the other hand, expression of *dio3* significantly ($P < 0.0001$) increased on Stage 1 (hatch) and was significantly impacted by Temperature ($P = 0.014$; Fig. 3Q-R) when compared in developmental time, as well as significantly affected by the Temperature \times Age interaction ($P < 0.0001$) when compared in real time. Thus, the model was decomposed into a series of reduced ANOVA models to determine the effect of Temperature for each Age (Fig. 3S) and of Age for each Temperature (Fig. 3T-W). Significant differences in gene expression of *dio3* among temperatures occurred throughout development on 0 ($P = 0.030$), 2 ($P = 0.019$), 10 ($P = 0.044$), 12 ($P < 0.0001$) and 14 dph ($P = 0.006$; Fig. 3S). Larval Age significantly influenced gene expression of *dio3* when larvae were reared at 16, 18 and 20°C ($P < 0.0001$; Fig. 3T-V), but not at 22°C ($P = 0.056$; Fig. 3W). The relationships between Age and *dio3* expression can be explained by quadratic parabola regressions at all temperatures investigated (Fig. 3T-W).

3.3.2. Thyroid hormone receptors

In developmental time, expression of *thraA* significantly ($P < 0.0001$) increased on Stage 2 (teeth formation) and 3 (first-feeding), while no significant ($P = 0.167$) effect of Temperature was detected (Fig. 4A-B). In real time, gene expression of *thraA* was significantly affected by the Temperature \times Age interaction ($P = 0.039$). Thus, the model was as above decomposed into a series of reduced ANOVA models to determine the effect of Temperature for each Age (Fig. 4C) and of Age for each Temperature (Fig. 4D-G). Significant differences in gene expression of *thraA* among

temperatures occurred throughout development on 2 ($P = 0.033$) and 8 dph ($P = 0.016$; Fig. 4C). Larval Age significantly influenced gene expression of *thraA* and relationships between Age and *thraA* expression can be explained by linear regressions at 16°C, 20°C and 22°C (Fig. 4D, F and G), or by a sigmoidal cubic regression at 18°C (Fig. 4E).

Similar to *thraA*, expression of *thraB* significantly ($P < 0.0001$) increased on Stage 2 (teeth formation) and 3 (first-feeding), while no significant ($P = 0.267$) effect of Temperature was detected (Fig. 4H-I). In real time, gene expression of *thraB* was significantly affected by Temperature ($P = 0.006$; Fig. 4J) and Age ($P < 0.0001$; Fig. 4K), while no significant Temperature \times Age interaction ($P = 0.147$) was detected. Gene expression of *thraB* significantly increased at 22°C (Fig. 4J) and significantly increased (up to 5-fold) throughout ontogeny with increasing larval Age (Fig. 4K). The relationships between Age and *thraB* expression were best explained by linear regressions at 16°C, 20°C and 22°C (Fig. 4L, N and O), or by a sigmoidal cubic regression at 18°C (Fig. 4M).

In contrast to all the other *thrs*, expression of *thrβA* significantly ($P = 0.001$) decreased from Stage 1 (hatch), while no significant ($P = 0.098$) effect of Temperature was detected (Fig 5A-B). In real time, gene expression of *thrβA* was not significantly affected by Temperature ($P = 0.342$; Fig. 5C) and no Temperature \times Age interaction ($P = 0.940$) was detected. On the contrary, a significant ($P = 0.0003$) effect of Age was detected (Fig. 5D). The relationships between Age and *thrβA* expression were best explained by linear regressions at 18°C and 22°C (Fig. 5F, H). No significant relationships between Age and *thrβA* expression were detected at 16 and 20°C (Fig. 5E, G).

Similar to *thras*, expression of *thrβB* significantly ($P < 0.0001$) increased on Stage 2 (teeth formation) and 3 (first-feeding), while no significant ($P = 0.203$) effect of Temperature was detected (Fig. 5I-J). In real time, gene expression of *thrβB* was significantly affected by Temperature ($P = 0.012$; Fig. 5K) and larval Age ($P < 0.0001$; Fig. 5L), while no significant

Temperature \times Age interaction ($P = 0.296$) was detected. Gene expression of *thr β B* significantly increased at 16°C and 22°C (Fig. 5K), while significantly increased (up to 6-fold) throughout ontogeny with increasing larval Age (Fig. 5L). The relationships between Age and *thr β B* expression were best explained by linear regressions at 16°C and 22°C (Fig. 5M, P), or by quadratic parabola regressions at 18°C and 20°C (Fig. 5N, O).

4. Discussion

This study aimed at enhancing our knowledge on the molecular mechanisms controlling European eel early life development. In fish, it has been suggested that THs play a role during the transitional period towards exogenous feeding and that *thr* expression can be affected by food deprivation (Raine et al., 2005). This study focused solely on the endogenous feeding period, where we investigated the expression patterns of genes encoding for THR and deiodinases from hatching until first-feeding. Generally and within the thermal tolerance limits of each particular species, increasing temperature accelerates all biochemical processes, causing a shift in early fish development (Martell et al., 2005; Politis et al., 2014b) and gene expression can follow a similar pattern (Politis et al., 2017). In this study, we observed that the warmer the temperature the earlier the expression response of a specific target gene. Thus, even though the expression profiles often appeared very similar and only shifted with temperature (when compared in real time), they were affected (in real and/or developmental time) by larval age (or stage), temperature, and/or their interaction.

In more detail, fish ELH has been correlated to thyroid hormone metabolism, which is under the control of the hypothalamus-pituitary-thyroid axis, where the thyroid gland is synthesizing TH (Jarque and Piña, 2014). This is regulated by the thyroid stimulating hormone (thyrotropin), which

is released by the pituitary as a consequence of the thyrotropin-releasing hormone from the hypothalamus (MacKenzie et al., 2009). TH is synthesized as a precursor (T4) with weak bioactivity and can be converted into active hormones (T3) or inactive metabolites (reverse-T3 or T2) by three transmembrane proteins, the iodothyronine deiodinases (DIO1-3), in peripheral tissues. They selectively remove iodine moieties towards the active forms through the outer and the inactive forms through the inner ring deiodination processes (Jarque and Piña, 2014). Here, we *inter alia* identified and followed the expression patterns of all three iodothyronine deiodinase genes (*dio1-3*) during early (yolk-sac) larval stages of experimentally reared (from hatch until first-feeding) European eel. Interestingly, expressions of *dio2* and *dio3* were elevated at hatch, potentially due to maternal origin (Castillo et al., 1993; Olsen and Press, 1997; Huttenhuis et al., 2006; Swain and Nayak, 2009; Lee et al., 2013), or possibly representing their involvement in specific processes around hatching. Thus, it would be of interest to follow their expression patterns (and the consequences of potential variations) also during embryogenesis and embryonic development, in order to elucidate their role during this sensitive life period. Furthermore, the expression slowly decreased during the first couple of dph until a clear sigmoidal or exponential increase was observed, respectively. For *dio1*, we did not observe a similarly clear pattern in all thermal regimes.

Deiodination in the peripheral tissues of fish has been linked to several functional and vital processes, while showing sensitivity to environmental organic contaminants (Adams et al., 2000; Couderc et al., 2016), as well as physical environmental factors, such as pH (Mol et al., 1998), temperature (Adams et al., 2000), salinity (Orozco et al., 2002), and/or light (Comeau et al., 2000; Wambiji et al., 2011). Temperature has been shown to influence the outer and/or the inner ring deiodination of species, such as rainbow trout, *Oncorhynchus mykiss* (Johnston and Eales, 1995), blue tilapia, *Oreochromis Aureus* (Mol et al., 1997), Atlantic cod, *Gadus morhua* (Cyr et al., 1998; Comeau et al., 2000), American plaice, *Hippoglossoides platessoides* (Adams et al., 2000) and

zebrafish (Little et al., 2013). In the present study, we experimentally showed that temperature had a significant effect (in real and/or developmental time) on gene expression related to deiodination activities during early larval development of European eel. Moreover, we showed that the expression of all deiodination genes are significantly affected by the age or stage of eel larvae. Within the thermal tolerance limits, we commonly observed elevated expression towards and within the first-feeding window, which probably corresponds to a timing of refinement for organogenesis (e.g. brain, liver, gastro intestinal tract, etc.) and/or specific functional tissue (e.g. teeth, eyes, etc.), in order to ensure optimal transition from endogenous to exogenous feeding (Sørensen et al., 2016; Butts et al., 2016).

Thyroid hormones are moreover known to regulate the level of THR α s, which are members of the steroid hormone receptor superfamily (Evans, 1988). Two subtypes, with isoforms within the subtypes, are found to be products of distinct genes or splice variants of the same gene (Kawakami et al., 2013). In the present work, we have identified and cloned the full CDS of two *thra* (*thraA* and *thraB*) and two *thr β* (*thr β A* and *thr β B*) gene forms in European eel larvae and followed their expression patterns from hatch to the first-feeding stage. All four European eel sequences contain the conserved DBD and LBD domains and two putative zinc fingers, providing evidence that they should be functional (see Supplementary material). The phylogenetic tree constructed, using several THR vertebrate sequences, reveals that European eel *thrs* are close to their fish orthologs, showing the highest homology (97-99%) with Japanese eel sequences (Kawakami et al., 2013). In *thraB*, however, an insertion of 22 amino acids was observed at the N-terminus part (A/B domain), which is missing in the corresponding Japanese eel sequence.

Similarly, to the genes involved in the deiodination process, *thrs* were affected by both, larval age, and temperature. Expression of *thr* genes increased during development, usually in a linear or an exponential fashion, with the exception of *thr β A* that decreased during development. Similar to

the expression of deiodinases, expression of *thrs* was usually high around the first-feeding stages, probably representing the involvement of the THRs towards and during the refinement of organogenesis and specific tissue for an optimal transition to exogenous feeding. Moreover, in real time, temperature was found to impact the expression pattern of *thr* genes. Generally, increasing temperature caused acceleration in development (Politis et al., 2017) and the warmer the temperature, the earlier the expression response of a specific target gene, potentially corresponding to the timing of specific ontogenic development. Interestingly, the expression of *thrβA* showed a slight increase at the first sampling points (hatch), which could theoretically be due to maternal origin (Castillo et al., 1993; Olsen and Press, 1997; Huttenhuis et al., 2006; Swain and Nayak, 2009; Lee et al., 2013), while it remained low during the rest of the investigated developmental period. This could imply that *thrβA* genes are not involved in ontogenic development of the ELH stages but potentially gain functionality during the later and/or adult stages of this species, as it has been presented for the related Japanese conger eel (Kawakami et al., 2003a,b) and Japanese eel (Kawakami et al., 2013).

In conclusion, we cloned and characterized *thr* sequences, followed the expression pattern of *thrs* and *dios*, and showed that temperature affected the expression of those genes in artificially produced ELH stages of European eel, reared in different thermal regimes from hatch until first-feeding. We identified 2 subtypes of *thr* (*thra* and *thrβ*) with 2 isoforms each (*thraA*, *thraB*, *thrβA* and *thrβB*) and 3 subtypes of deiodinases (*dio1*, *dio2* and *dio3*). All *thr* genes identified, show high similarity to other mammalian, bird, amphibian or fish species, with highest similarity to the closely related Japanese eel. We can confidently state that all genes investigated in this study, involved in the mediation of TH action, were significantly affected (in real and/or developmental time) by larval age, temperature, and/or their interaction. Finally, we demonstrate that *thrs* and *dios* show sensitivity to temperature and are involved in and during early life development of European eel.

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Table 1: Sequences of European eel, *Anguilla anguilla* primers used for amplification of genes by qRT-PCR. Primers were designed from cloned THR cDNA sequences and other sequences available in GenBank Nucleotide, the European eel transcriptome database (EeelBase 2.0, <http://compgen.bio.unipd.it/eeelbase/>) or the eel genome website (<http://www.zfgenomics.com/sub/eel>). The table lists accession number and corresponding database of target gene sequences.

Full name	Abbreviation	Databases	Accession Numbers	Primer sequence (5' - 3') (F: Forward; R: Reverse)
Deiodinase 1	<i>dio1</i>	EeelBase 2.0	Eeel2-c186	F: AGCTTTGCCAGAACGACTGT R: TTCCAGAACTCTTCGCACCT
Deiodinase 2	<i>dio2</i>	Eel genome website	g12347	F: GAAGAGGAGGATCGCCTACC R: GCACTCTACCTCCGTCCAAA
Deiodinase 3	<i>dio3</i>	EeelBase 2.0	Eeel-c22164	F: TACGGGGCGTATTTTGAGAG R: GCTATAACCCTCCGGACCTC
Thyroid Hormone Receptor alpha A	<i>thraA</i>	GenBank Nucleotide	KY082904	F: GCAGTTCAACCTGGACGACT R: CCTGGCACTTCTCGATCTTC
Thyroid Hormone Receptor alpha B	<i>thraB</i>	GenBank Nucleotide	KY082905	F: GAAGCCTTCAGCGAGTTCAC R: ACAGCCTTTCAGGAGGATGA
Thyroid Hormone Receptor beta A	<i>thrβA</i>	GenBank Nucleotide	KY082906	F: AGGAACCAATGCCAAGAATG R: GCCTGTTCTCCTCAATCAGC
Thyroid Hormone Receptor beta B	<i>thrβB</i>	GenBank Nucleotide	KY082907	F: GAAGACTGAGCCCTGAGGTG R: AGGTAATGCAGCGGTAATGG
Elongation Factor 1-alpha	<i>ef1a</i>	GenBank Nucleotide	EU407824.1	F: CTGAAGCCTGGTATGGTGGT R: CATGGTGCATTTCACAGAC
Ribosomal 18S RNA	<i>18s</i>	EeelBase 2.0	eeel2_s7245	F: AGAGCAGGGGAAGTACTGACTGA R: ACCTGGCTGTATTTGCCATC
Ribosomal 40S RNA	<i>40s</i>	GenBank TSA	GBXM01005349.1	F: TGACCGATGATGAGGTTGAG R: GTTTGTGTGCCAGACCGTTG

ACCEPTED MANUSCRIPT

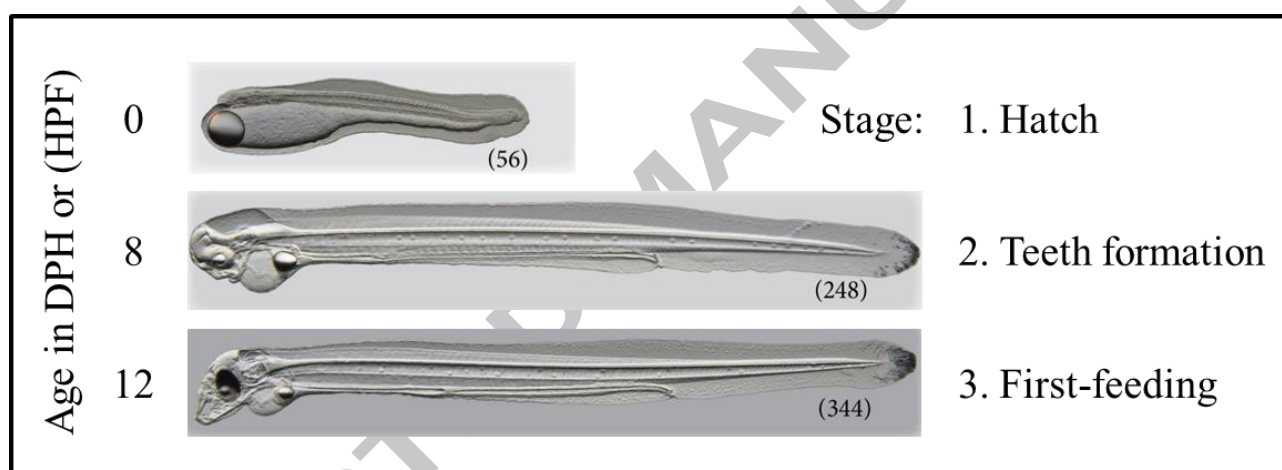
Fig. 1: Stage specific European eel larval development at 18°C in days post hatch (DPH) and hours post fertilization (HPF). Stage 1 represents hatching, Stage 2 represents teeth formation, and Stage 3 represents first-feeding.

Fig. 2: Phylogenetic relationships among thyroid hormone receptors (THRs) of four vertebrate classes were inferred using the Neighbor-Joining method. Fish orders are in italics. The sequences of European eel, *Anguilla anguilla* obtained in this work are underlined. The percentages of replicate trees in which the associated taxa are clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. Phylogenetic analysis was conducted in MEGA6. S or L in the sequence's name stands for small and large protein forms. The mention tr indicates sequences of truncated proteins. Partial sequences have been omitted.

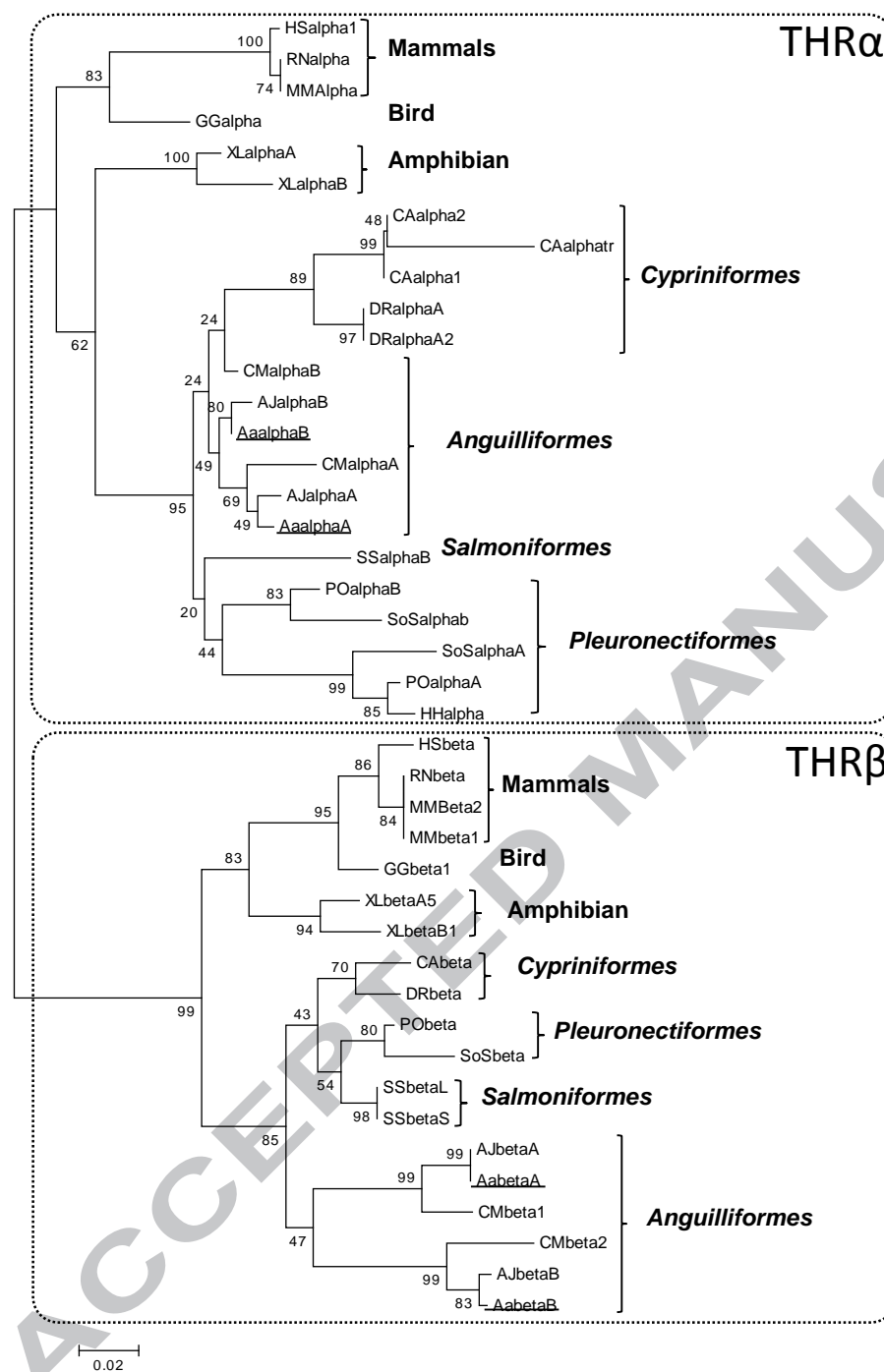
Fig. 3: Effect of temperature on larval European eel, *Anguilla anguilla* gene expression of *dio1* (A-B), *dio2* (I-J) or *dio3* (Q-R) at specific developmental Stages (1: hatch, 2: teeth formation and 3: first-feeding) and *dio1*(C-D), *dio2* (K-L) or *dio3* (S) in real time, as well as effect of age on *dio1* (E-H), *dio2* (M-P) or *dio3* (T-W) expression at each temperature. Data points with an asterisk (*) were not included in the statistical model due to insufficient sample size. Values represent means (\pm SEM) among 4 crosses at each temperature. Means were contrasted using the Tukey-Kramer method and treatments with the same letters are not significantly different ($P > 0.05$).

Fig. 4: Effect of temperature on larval European eel, *Anguilla anguilla* gene expression of *thraA* (A-B) or *thraB* (H-I) at specific developmental stages (1: hatch, 2: teeth formation and 3: first-feeding) and *thraA* (C) or *thraB* (J-K) in real time, as well as effect of age on *thraA* (D-G) or *thraB* expression (L-O) at each temperature. Data points with an asterisk (*) were not included in the statistical model due to insufficient sample size. Values represent means (\pm SEM) among 4 crosses at each temperature. Means were contrasted using the Tukey-Kramer method and treatments with the same letters are not significantly different ($P > 0.05$).

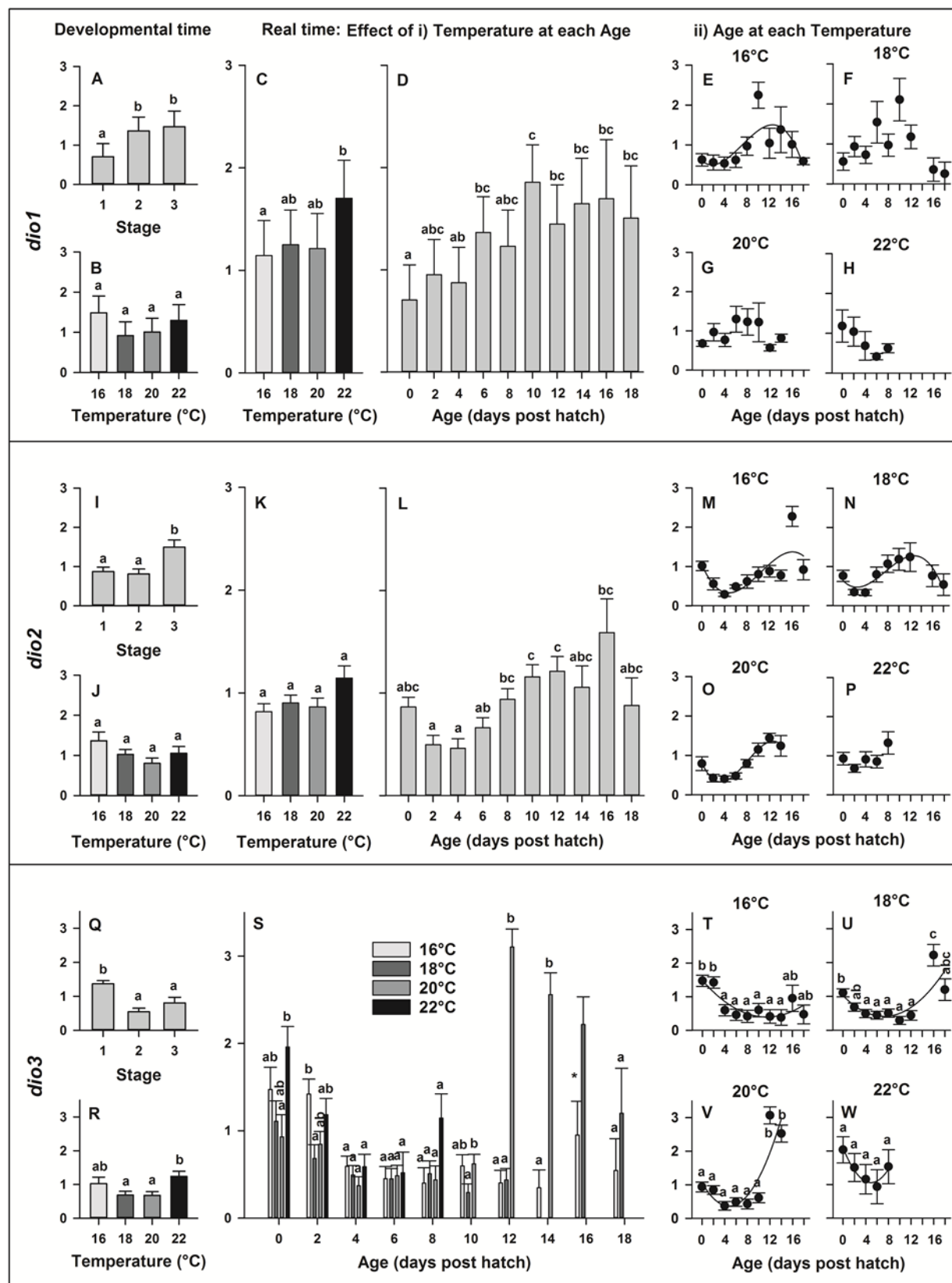
Fig. 5: Effect of temperature on larval European eel, *Anguilla anguilla* gene expression of *thrβA* (A-B) or *thrβB* (I-J) at specific developmental stages (1: hatch, 2: teeth formation and 3: first-feeding) and *thrβA* (C-D) or *thrβB* (K-L) in real time, as well as effect of age on *thrβA* (E-H) or *thrβB* expression (M-P) at each temperature. Values represent means (\pm SEM) among 4 crosses at each temperature. Means were contrasted using the Tukey-Kramer method and treatments with the same letters are not significantly different ($P > 0.05$).



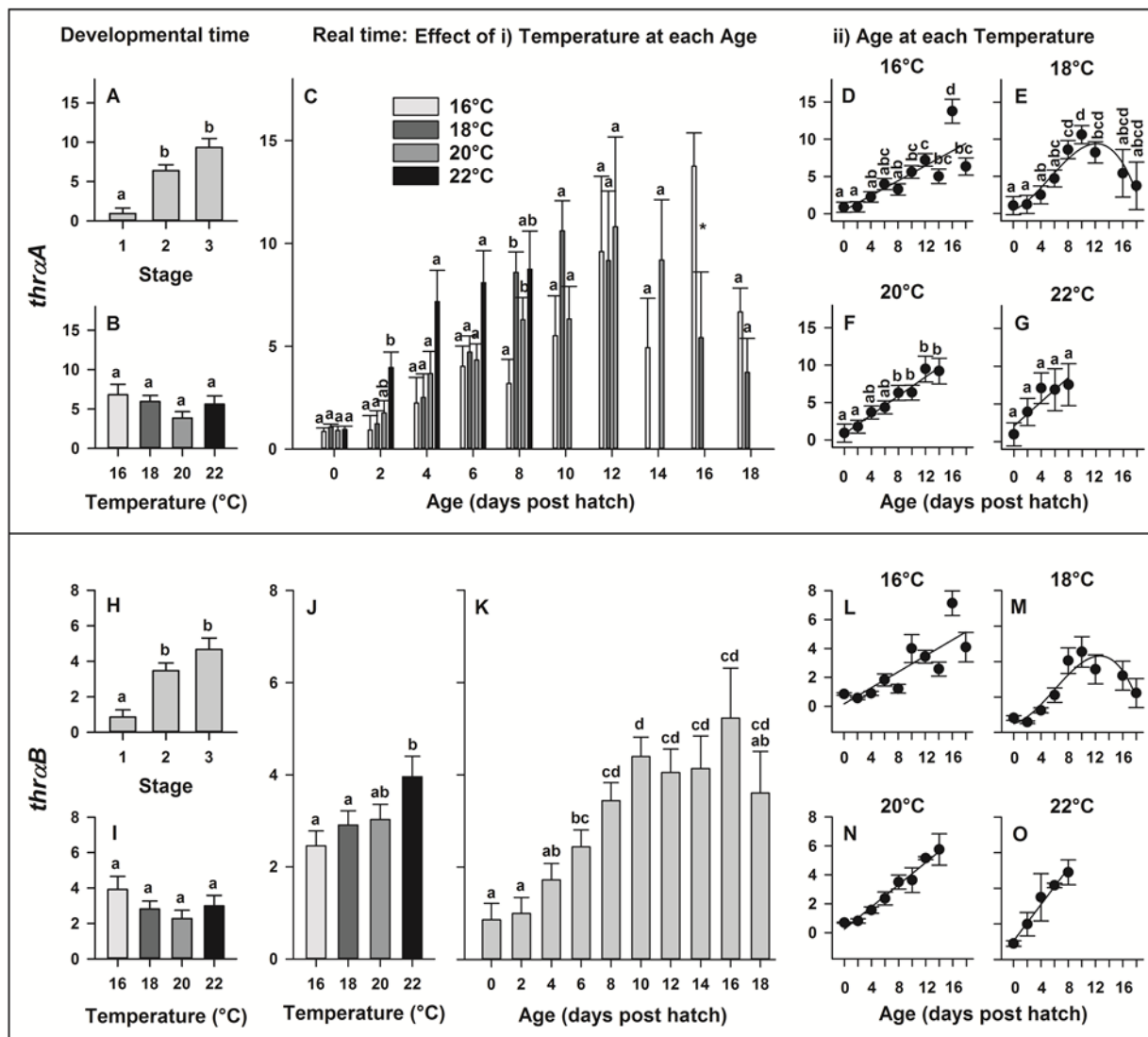
Figure_1

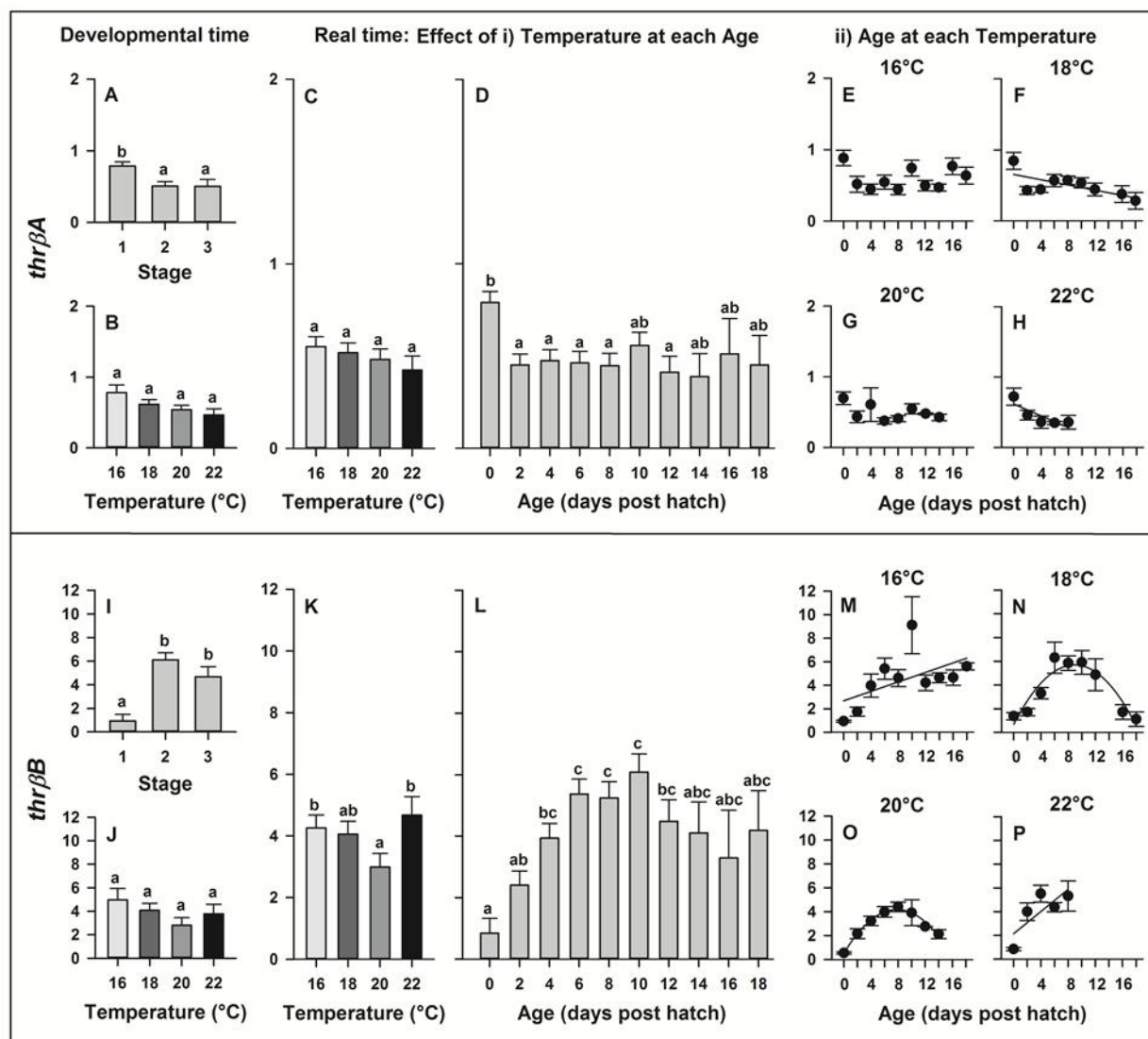


Figure_2



Figure_3





Figure_5

Highlights:

- 1) European eel larvae were produced via assisted reproduction in captivity.
- 2) Thyroid hormone pathway (*thraA-B*, *thrβA-B* and *dio1-3*) related genes identified.
- 3) European eel thyroid hormone receptor sequences were cloned and characterized.
- 4) Temperature, larval age and/or their interaction affected gene expression.
- 5) Vertebrate thyroid hormone receptor sequences were phylogenetically analyzed.